THE EFFECTS OF DIFFERENT HALOGENATED ALKYL AMINES ON THE DIVISION OF SEA URCHIN EGGS[1]

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With a view to obtaining some insight into the cytological effects of a representative series of nitrogen mustards, 27 compounds, mostly β-chloroethyl amines, were tested for their ability to block or retard the cleavage of sea urchin eggs.

With such eggs, each exposed directly to the experimental solution, there is unexcelled opportunity to study the response of the cell to the mustard without concern for effects of a multicellular organism on the mustard, and on the response of the cell. With regard to mitosis, the uniform, controllable division of the eggs makes possible the application of the agent to any phase of cleavage, and ready analysis of alterations in each phase.

Materials and Methods

For the over-all survey, the eggs of *Arbacia punctulata* were used. Some supplementary studies employed the eggs of *Tripneustes esculentus,* and for detailed observations of the course of events during inhibition of mitosis, the transparent eggs of *Lytechinus variegatus* were used.

For routine comparison of activities, exposure was begun 10–13 minutes after fertilization. Retardation was measured by making a count of 50 eggs 4 to 10 times during the first cleavage and again during the second. The points were connected by straight lines to determine the approximate time of 50 per cent cleavage (*cf.* Fig. 1), and comparison was made between the treated and the control from the same lot of eggs.

Curves for duplicate controls usually gave identical 50 per cent cleavage times, and they seldom differed by more than two minutes, so statistical analysis was not needed. The eggs were followed until they formed plutei, and a record was kept of the time at which they became blastulae and gastrulae. The ranges of response were condensed into groups described in connection with Table II.

The compounds (Table I) were dissolved in sea water and were added to the eggs within two minutes of dissolving. They were supplied to the Sloan-Kettering Institute by Parke-Davis and Company (A); Toxicity Laboratory, The University of Chicago (B); Dr. Stein, Rockefeller Institute (C); Merck and Company (D); Eli Lilly and Company (E); and American Cyanamid Company (F).

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Results and Discussion

Arbacia

The nature of the response of dividing eggs to nitrogen mustard at moderately effective doses is shown in Figure 1. Lower concentrations caused less retardation or affected only the second division or even later stages, while higher concentrations prevented division in all or part of the population.

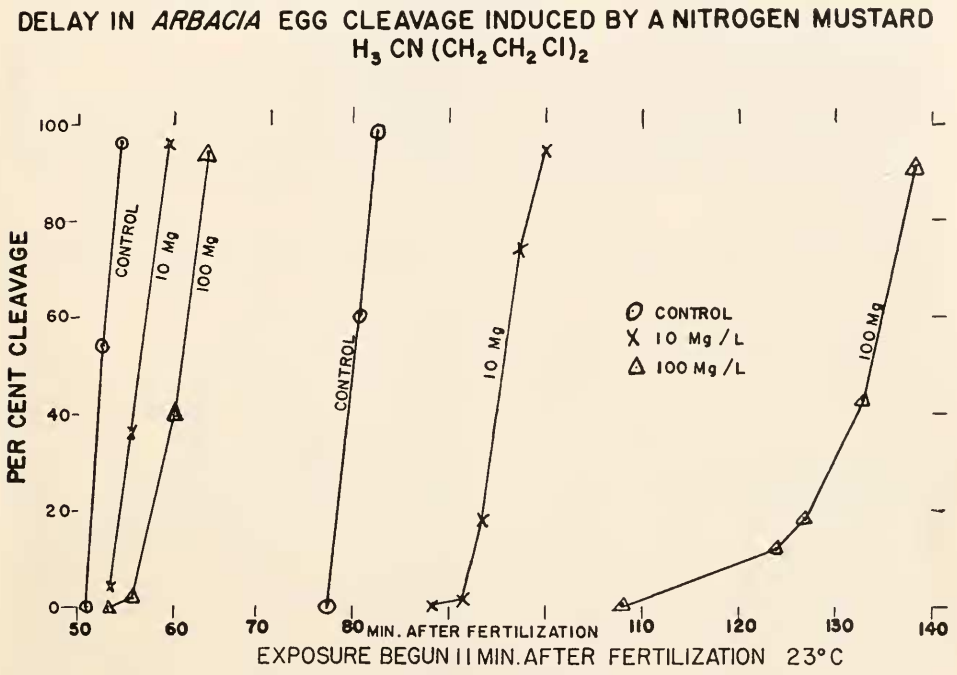
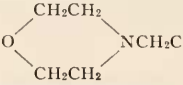
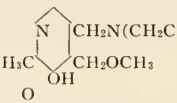
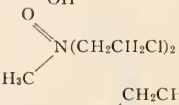
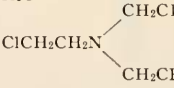
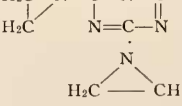
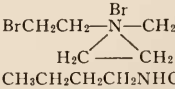


Figure 1. The three curves on the left represent first cleavage, the others second cleavage.

The different degrees of retardation and blocking of Arbacia-egg cleavage are shown in Table II, beginning with the most active mustard at the top. Concentrations are expressed as millimols per liter. The grades of effect are the same as those used to quantitate effects of carbamates (Cornman, 1950). Where development was not completely blocked, retardation of the first cleavage was determined from graphs like Figure 1 and then placed in one of three grades: 100% or more, 10% to 99%, or less than 10% increase in the time required to reach 50% first cleavage as compared with the controls. Weaker effects were detected as retardation of second cleavage or of embryogenesis. At 23° ± 0.5° C., the temperature used for all Arbacia experiments, 50 per cent first cleavage was reached usually at 52–53 minutes, but in one experiment was as early as 50 minutes and in another as late as 61 minutes. Second cleavage reached 50 per cent at 84–85 minutes, with extremes at 80 and 93 minutes.

TABLE I

Names and structures of compounds studied

No.	Name	Structure	Source
		β-chloroethylamines	
1	*β*-chloroethyl amine. HCl	$NH_2CH_2CH_2Cl \cdot HCl$	A
2	N-(*β*-chloroethyl)-morpholine		B
3	Dibenzyl-*β*-chloroethyl amine. HCl	$(\text{Ø}CH_2)_2NCH_2CH_2Cl \cdot HCl$	B
4	*Bis*-(*β*-chloroethyl)-amine. HCl	$NH(CH_2CH_2Cl)_2 \cdot HCl$	A
5	Methyl-*bis*-(*β*-chloroethyl)-amine. HCl	$H_3CN(CH_2CH_2Cl)_2 \cdot HCl$	B
6	Propyl-*bis*-(*β*-chloroethyl)-amine	$CH_3CH_2CH_2N(CH_2CH_2Cl)_2$	B
7	*n*-amyl-*bis*-(*β*-chloroethyl)-amine. HCl	$CH_3(CH_2)_4N(CH_2CH_2Cl)_2 \cdot HCl$	D
8	*n*-hexyl-*bis*-(*β*-chloroethyl)-amine. HCl	$CH_3(CH_2)_5N(CH_2CH_2Cl)_2 \cdot HCl$	D
9	*γ*-phenylpropyl-*bis*-(*β*-chloroethyl)-amine. HCl	$\text{Ø}CH_2CH_2CH_2N(CH_2CH_2Cl)_2 \cdot HCl$	D
10	*p*-octylphenoxyethyl-*bis*-(*β*-chloroethyl)-amine. HCl	$C_8H_{17}\text{Ø}OCH_2CH_2OCH_2CH_2N(CH_2CH_2Cl)_2 \cdot HCl$	D
11	3-*bis*-(*β*-chloroethyl)-aminom hyl-4-methoxy-methyl-5-hydroxy-6-methyl-pyridine. 2HCl	 2HCl	D
12	Methyl-*bis*-(*β*-chloroethyl)-amine oxide. HCl		D
13	N,N′-*bis*-(*β*-chloroethyl)-1,4-piperazine. HCl		D
14	*Tris*-(*β*-chloroethyl)-amine. HCl	$N(CH_2CH_2Cl)_3 \cdot HCl$	B
15	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-ethylene-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CH_2N(CH_2CH_2Cl)_2 \cdot 2HCl$	B
16	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-propane-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CH_2CH_2N(CH_2CH_2Cl)_2 \cdot 2HCl$	B
17	N,N,N′,N′-*tetrakis*-(*β*-chloroethyl)-*β*-chloropropane-diamine. 2HCl	$(ClCH_2CH_2)_2NCH_2CHClCH_2N(CH_2CH_2Cl)_2 \cdot 2HCl$	B
18	Composition uncertain. Probably a polymer of *bis*-(*β*-chloroethyl)-amine with M.W. 764	$H_2[HCN(CH_2CH_2Cl)_2]_n \cdot nHCl$	D
		Other Chloroalkylamines	
19	Ethyl-*γ*-chloropropyl-*β*-chloroethylamine. HCl	$ClCH_2CH_2N(CH_2CH_3)CH_2CH_2CH_2Cl \cdot HCl$	E
20	*Tris*-(*β*-chloro-*n*-propyl)-amine. HCl. $\frac{1}{2}H_2O$	$N(CH_2CHClCH_3)_3 \cdot HCl \cdot \frac{1}{2}H_2O$	D
21	Triethylenimino-*s*-triazine		F
		Other Haloalkylamines	
22	Methyl-*bis*-(*β*-bromoethyl)-amine. HBr	$CH_3N(CH_2CH_2Br)_2 \cdot HBr$	D
23	N,N′-(*β*-bromoethyl)-N,N′-(*β*-ethylene-imonium-bromide)-ethylene-diamine		D
24	*n*-butyl-*β*-fluoroethylamine. HCl	$CH_3CH_2CH_2CH_2NHCH_2CH_2F \cdot HCl$	D
25	*n*-butyl-*bis*-(*β*-fluoroethyl)-amine. HCl	$CH_3CH_2CH_2CH_2N(CH_2CH_2F)_2 \cdot HCl$	D
		Substituted Ammonium Chlorides	
26	*bis*-(*β*-chloroethyl)-dimethyl-ammonium chloride	$(CH_3)_2NCl(CH_2CH_2Cl)_2$	B
27	tetra-(*β*-chloroethyl)-ammonium chloride	$(ClCH_2CH_2)_2NCl(CH_2CH_2Cl)_2$	C

This series of mustards divides roughly into two groups, the majority blocking cleavage at a few millimols per liter, and retarding the first division at the tenths or hundredths millimolar level (down to No. 13, Table II). About half of this group, including the more typical nitrogen mustards (8, 15, 7, 9, 14, 6, 22), are somewhat more active than the others, increasing cleavage time 100 per cent or more at 0.3–0.5 mM/L and 10–99 per cent at 0.03–0.05 mM/L. The quick effectiveness of *n*-hexyl-*bis*-(β-chloroethyl)-amine (No. 8), preventing all division at 0.38 mM/L, suggests that the six-carbon chain is about optimal for penetration, as against the longer and shorter groups tested.

TABLE II

Relative potencies (mM/L) of halogenated alkyl amines in preventing or retarding Arbacia egg cleavage

Compound No.	Millimolarity required to induce:						
	Complete block (Aa)	Retardation					No effect
		First cleavage			Second cleavage (Cd)	Embryo (D)	
		100% (Ca)	10–99% (Cb)	<10% (Cc)			
8	0.38		.038	.019 .008			
15	2.61	.261	.026				
7	4.02	.402	.040				
9	3.37	.337	.034				
14	4.15	.415	.042				
6	5.44	.544	.544	.054			
22	3.07		.307 .031				
20	3.43		.343	.034			
12			.481	.048			.005
5	5.21		.521	.052			
19			4.52 .452	.045			
10	2.20			.220	.022		
18		1.31		.131	.013		
13	4.05				.405		
3				.338			.034
2			6.85	.685			
1			8.62	.862 .086			
27			3.29				.329
4			11.17	5.59 .559 .056			
26						4.83 .483	
23			20.49				2.05

Aa: No nuclear or cytoplasmic division. C: The time between fertilization and 50% first cleavage increased by 100% (Ca), by 10–99% (Cb), or by less than 10% (Cc). Cd: First cleavage not retarded but second cleavage retarded. D: First two cleavages not retarded but the blastula, gastrula, or pluteus retarded.

Two potent mustards deviate from the typical structural pattern. Methyl-*bis*-(β-chloroethyl)-amine oxide (No. 12) has its nitrogen occupied by oxygen in addition to the alkyl chains. Attachment of the β-chloroethyl radicals to separate nitrogens (No. 13) also forms an active molecule, as pointed out by Goldacre, Loveless and Ross (1949). A terminal chlorine (No. 19) or a β-chlorine (No. 20) in a propyl chain appears to function as well as an ethyl β-chlorine, although Haddow, Kon and Ross (1948) find the γ chlorine inactive in chloroalkylaryl amines. The poor performance of No. 4 is under suspicion, particularly since these compounds hydrolyze readily in the moist seaside atmosphere. A fresh sample was not available for re-checking.

Mustards with only a single β-chloroethyl group (Nos. 1, 2, 3) are relatively inactive, as reported by Haddow, Kon and Ross (1948). On the other hand, addition of a third β-chloroethyl group (14) or even a second substituted nitrogen (15) does not confer outstanding potency on the molecule.

Tripneustes

Experiments with another species at the same temperature show some differences in response, and, because the egg is unpigmented, yield somewhat more in the way of cytological detail.

For comparison, the column headings used for Arbacia (Table II: Ca, Cb, etc., indicating the extent of retardation) are similarly used for Tripneustes in Table III. An additional category, *Incomplete block,* encompasses more severe effects where less than 5 per cent of the eggs divided (b) or where they did not divide at all, but unlike completely blocked eggs, they formed temporary furrows or underwent nuclear division in the absence of cytoplasmic division (Ba). Comparison is made at the time of 50 per cent cleavage which was at 86–101 minutes for the first, and 131–182 minutes for the second division at 23°, or 65–82 minutes for the first and 101–122 minutes for the second at 25°–26° C.

Some distinctive cytological responses were observed in the living eggs. In 0.521 mM of methyl-*bis*-(β-chloroethyl)-amine (No. 5), asters and spindle remained distinct during the 1½ hours that cleavage was delayed and persisted even longer in eggs which did not divide, eventually producing tetranucleate eggs at 6 hours. At 0.052 mM, 60% of the eggs furrowed, but the furrows retracted in half of these. Persistent, often multiple, mitotic figures were also seen in compounds 15 and 24.

In comparing the effects of Nos. 5, 14 and 15 with the less severe damage to Arbacia eggs, it should be kept in mind that the length of exposure before the first cleavage is nearly twice as long in Tripneustes, which divides at about 1½ hours as against 50–60 minutes for Arbacia at 23° C.

No. 24 has only a single fluoroethyl radical, and, as we have seen from Arbacia studies, such "hemi-mustards" are relatively ineffective. However, No. 24, although having both β-fluoroethyl groups, is considerably less toxic than the chloroethylamines. The difference cannot as yet be assigned entirely to the halogen inasmuch as the first five (14, 15, 16, 17, 5) were tested at Bermuda at 23° C. and the last three (11, 25, 24) at Bimini at 25°–26° C. There are also differences among different experiments with the same compound, as shown by diverse severity of effects from identical concentrations of Nos. 15 and 24. These dif-

ferences arise from differences in the numbers of eggs in separate experiments and probably from differences in the individual urchins.

Lytechinus

In this species 50 per cent cleavage is reached at 46–61 minutes and the second at 70–83 minutes at 25°–27° C.

Triethylenimino-s-triazine

Triethylene melamine is a name commonly applied to compound No. 21. Although lacking the β-halogen, it already carries the imine rings which are formed by some chloroethylamines in solution (Golumbic *et al.*, 1946) and it resembles nitrogen mustards at least in its ability to retard leukemia (Burchenal, *et al.*, 1950) and solid tumors (Lewis and Crossley, 1950), and its effects on cell division (Rose, Hendry and Walpole, 1950).

TABLE III

Relative potencies (mM/L) of halogenated alkyl amines in preventing or retarding Tripneustes egg cleavage

Compound No.	Millimolarity required to induce:								
	Block			Retardation					No effect
	Complete (Aa)	Incomplete		First cleavage			Second cleavage (Cd)	Embryo (D)	
		(Ba)	(Bb)	100% (Ca)	10–99% (Cb)	<10% (Cc)			
14	0.415			0.042		0.004			0.0004
15		0.261		0.261	0.026	{0.026 0.003			{0.003 0.0003
16			0.252		{0.025 0.003				0.0002
17					{0.232 0.023		0.002		0.0002
5				0.521	0.052	0.005			0.0005
11	2.630				{0.500 0.263 0.250		0.025		
25					4.975	0.498		0.050	
24					6.430	0.643	6.430	0.064	

Aa: No cytoplasmic or nuclear division. Ba: Evanescent furrows or nuclear division in the absence of cytoplasmic division. Bb: Less than 5% of the eggs divided. C: The time between fertilization and 50% first cleavage increased by 100% (Ca), by 10–99% (Cb), or by less than 10% (Cc). Cd: First cleavage not retarded but second cleavage retarded. D: First two cleavages not retarded but the blastula, gastrula, or pluteus retarded.

At 9.8 mM/L it retarded the first cleavage 35–42 minutes (Cb) and prevented completion of the second cleavage. Half this dose retarded the first cleavage 25–30 minutes (Cb) and the second cleavage 1–2 hours. The second division was remarkably unequal, producing some blastomeres ¼ and ⅓ the diameter of their sisters. At 0.49 mM/L the first cleavage was retarded 4 minutes and the second 29

minutes, but the embryos died before becoming blastulae. Retardation of the first cleavage was one minute and the second 9 minutes at 0.049 mM/L. These became abnormal gastrulae.

Triethylenimino-*s*-triazine thus resembles the mustards in producing extreme retardation at comparable doses, but is unlike them in that concentrations which produce slight retardations are eventually lethal.

Dose-effect relationships with HN2

For the more detailed study of dose-effect relations and cytological effects, methyl-*bis* (β-chloroethyl)-amine (Compound 5, often called HN2) was chosen as a typical mustard. The Lytechinus egg was used because the nucleus and mitotic figure can be clearly observed in the living egg.

TABLE IV

Relationships between concentration of HN2 and inhibition of cleavage in Lytechinus eggs

	mM/L	% First cleavage		Retardation (minutes)		% Increase in cleavage time	
		Count	% Inhib.	1st cl.	2nd cl.	1st cl.	2nd cl.
Expt. III	8.320	2	98				
	4.160	22	76				
	2.080	46	49	40	210	63	248
	1.040	68	24	32	70	50	83
Expt. I	2.080	36	60	38	180	68	254
	1.040	90	0	9½	54½	17	66
	0.521			4	37	7	45
	0.260			0	21		25
	0.130			0	15½		19
	0.065			0	7		8
	0.032			0	1½		2

Expt. III: Control first cleavage 63½ min.; second cleavage 84½ min.
Expt. I: Control first cleavage 55½ min.; second cleavage 82½ min.
Percentage inhibition based on 90% control cleavage in both experiments.

The percentages of Table IV show that the curve for blocking (2–8 mM) is steeper than for retardation (0.03–1.0 mM), suggesting that two mechanisms are involved. Quite possibly cytoplasmic division is affected separately at the higher concentrations, a separation which was visible in eggs where cleavage was suppressed but where the achromatic figure was still active and the nucleus sometimes divided. Higher doses were required to produce effects comparable to those obtained with Arbacia and Tripneustes. These experiments were run at Bimini at temperatures of 24.7°–25.8° C., so environmental as well as species differences may enter in.

"Prophase block"

Some investigators find that the prophase is particularly susceptible to mustard and other "radiomimetic" poisons. In sea urchin eggs, no one phase appeared

to be selectively hit. Rather, the entire mitotic sequence was slowed. In the experiments described below, the average temperature ranged from 24.7° C. to 26.7° C., with a maximum variation within each experiment of 0.2° C.

Eggs heavily poisoned (8.32 mM/L) 44–53 minutes before control 50 per cent cleavage, *i.e.*, early prophase, maintained a high percentage of intact nuclei, although the number fluctuated as if the nuclei were dissolved and reforming. In experiment III the first drop in numbers of nuclei was preceded by a period of persistent asters. The second decline, at 13 hours, showed no evidence of mitotic activity other than the disappearance of half of the nuclei—which then reappeared by 22 hours. At slightly lower doses (6.25 and 4.16 mM/L) an appreciable number of eggs divided, preceded by a normal dissolution of the nucleus. But nuclei also disappeared about the same time in 25–75 per cent of the eggs which did not divide. Moreover nuclei reappeared in only 25–75 per cent of the divided eggs. Hughes (1950) also reports reconstruction of nuclei in cells blocked at metaphase by nitrogen mustard.

Exposure begun when the eggs were in late prophase or in metaphase retarded the first cleavage, but permitted most of the eggs to divide. At 8.32, 6.25 and 4.16 mM/L the effects were the same as with doses earlier in the cycle, except that they appeared one cleavage later. Nuclei appeared in some of the blastomeres, and some of these then disappeared, usually coincident with cleavage. Most of the blastomeres resulting from the second and later cleavages did not form nuclei. A single nucleus appeared in about half of the eggs which did not divide. A small percentage of the one-cell and two-cell stages formed karyomeres instead of whole nuclei. Occasionally a blastomere became binucleate or underwent multiple cleavage.

Exposure to 6.25 mM/L at the end of the first mitotic cycle or early in the second produced the same phenomena one stage later. Nuclei appeared in most of the blastomeres. Half of them disappeared at a time when other eggs were fragmenting, and in experiment VI, at least (exposure 17 minutes before 50 per cent second cleavage), most of these nuclei had reappeared by 9 hours after fertilization. At the two-cell and four-cell stages, karyomeres appeared in from 6 to 100 per cent of the blastomeres, depending upon the experiment. Rarely a blastomere at the two-cell stage was binucleate. Exposure begun when some of the eggs had already divided twice yielded a nuclear cycle in the four-cell stage: nuclei reaching 64 per cent, then dropping to 34 per cent as some of the eggs divided, and finally disappearing from all eggs at 9½ hours, when a fourth of the eggs were disintegrating.

These data show that even when eggs are exposed in early prophase, and poisoned to the extent that the prophase is much prolonged, the nucleus nevertheless dissolves and often re-forms. These reformed nuclei may be the "prophases" observed by others. Exposure late in the mitotic cycle shows that eggs can be stopped after completion of the prophase, presumably at metaphase since subsequently many form a single nucleus. Karyomeres, binucleation and multiple cleavage testify to the varying degrees of disruption to which the telophase is subject.

If there were a prophase effect such as is found in the intact mammal, one would expect that at the time for the first prophase, or at least at the second prophase, the nucleus would fold up into a pycnotic mass and the whole cell would expire. Yet, the prophases appear in an orderly manner, the asters build up, and a spindle replaces the nucleus. Such a mitotic figure might persist for an hour and

a half, and eventually succeed in dividing the egg. Indeed, if any one phase is to be considered sensitive in the sea-urchin egg, it is telophase. Frequently the eggs almost divide and then the furrow regresses, leaving a binucleate egg.

Evidence for prophase damage by mustards comes mostly from experiments with intact animals (Dustin, 1947; Friedenwald and Buschke, 1948; Gillette and Bodenstein, 1946). It is not found in isolated cells such as Arbacia eggs (Hutchens and Podolsky, 1948) or tissue cultures (Hughes, 1950; Meier and Schär, 1947; Fell and Allsopp, 1949) or in some plants (Novick and Sparrow, 1950; Hohl, 1948). This suggests that systemic influences on mitosis be considered before different sensitivities of division phases be ascribed entirely to intrinsic properties of the mitotic cycle.

Synergism with urethan

Skipper (1949) has found a synergism between nitrogen mustards and urethan in decreasing the white cell count in mice. For comparison, eggs were exposed to 22.5 and 45 mM urethan, and 0.032 and 0.065 mM HN2 (No. 5) separately or combined.

Urethan alone at 22.5 mM retarded first cleavage 6 minutes, and second cleavage 6 minutes and retarded the gastrulae. HN2 alone at 0.032 mM retarded the second cleavage 1½ minutes and caused abnormal blastulae. Together they retarded the first cleavage one-half minute and the second cleavage three minutes and produced abnormal blastulae.

In urethan alone at 45 mM, only 12 per cent of the eggs had divided an hour after the controls divided, and the gastrulae were irregular. HN2 at 0.065 mM did not affect the first cleavage, but retarded the second 7 minutes, and produced abnormal blastulae. These doses combined gave 12 per cent cleavage an hour late, and irregular blastulae.

The effects are no more than the separate effects of the urethan on the early cleavages and HN2 on the larvae. The synergism observed in mice would appear to involve systemic effects which do not operate on isolated dividing cells. Further, the different patterns of effect and of dose-effect ratios indicate caution (already pointed out by Loveless and Revell, 1949) in grouping these poisons together as "radiomimetic" (Dustin, 1947).

Summary

1. The capacities of different mustards for blocking or delaying the cleavage of sea urchin eggs were compared by exposing the eggs of *Arbacia punctulata, Tripneustes esculentus* and *Lytechinus variegatus* to freshly prepared solutions continuously, beginning 10 to 13 minutes after fertilization.

2. At least two β-halo groups are necessary to confer the highest potency of the molecule. A third β-chloroethyl or a second *bis*-(β-chloroethyl)-amine group, or a non-chlorinated substitution on the nitrogen alters the activity of the molecule. β-chloropropyl or γ-chloropropyl can substitute for the β-chloroethyl radical. Methyl *bis*-β-bromine was slightly more active than its chlorine homologue and a fluorine congener was probably less active.

3. Environmental conditions have not been standardized in these experiments to permit an evaluation of differences in susceptibility of the different species.

4. In the range of 0.065–1.040 mM methyl-*bis*-(β-chloroethyl)-amine, retardation of second cleavage increases slowly with successive doublings of dose. Increasing the concentration beyond 1.040 mM rapidly decreases the percentage of eggs which divide.

5. The course of events in eggs exposed at the beginning of prophase, and later in the first and subsequent mitotic cycles points to a general slowing of mitosis. Any phase of mitosis can be blocked, depending upon the dose and time of exposure.

6. There is no synergism between urethan and methyl-*bis*-(β-chloroethyl)-amine when they are combined in threshold doses.

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